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METHODS IN VIROLOGY

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13 *Nucleic Acid Hybridization Technology and Detection of Proviral Genomes*

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I. Introduction

There has been a flood of techniques developed in the last few years to define sequence homology between species of nucleic acids. The power and versatility of these techniques are nowhere better displayed than in virus systems, especially those that have features of integration, proviral states, or other forms of latency of the viral genome or portions of it. Viral genomes, because of their uniqueness and limited complexity, lend themselves especially well to varied hybridization approaches, but there is no doubt that more complex genomes, including mammalian DNA, will become accessible to such analytic techniques; such efforts have already been initiated.

We have chosen to consider several techniques of nucleic acid hybridization in some detail rather than to catalog all the available methods. The prin-

ciples and general framework are similar in the many different systems that have been studied. Our focus is on hybridization systems that have proven of value in DNA virus systems. The same techniques can be used in RNA virus systems. However, they involve special problems, and we do not take them up in detail.

The purpose of this Chapter, the intent of which is practical rather than theoretical, is to deal with methods for the labeling, detection, quantitation, and determination of homology of viral DNA and RNA. We do this in the context of presentations of specific methods while indicating some of the practical theory behind the methods. We illustrate their uses and examine problems that are likely to be encountered in the laboratory and in interpretation of results. We also take up other selected methods of characterization of DNA that can fill an accessory role in hybridization approaches.

The topics included are preparation and radiolabeling of nucleic acids used as probes, DNA–DNA and RNA–DNA hybridization with immobilized nucleic acids, cytohybridization, and methods of kinetic analyses of DNA–DNA and RNA–DNA hybridization in solution. Finally, we present a limited discussion of the use of restriction endonuclease technology especially as it applies to blot-transfer techniques of nucleic acid hybridization and to the use of defined fragments of DNA as special probes. We finish with a consideration of heteroduplex technology, including R-loop technique. The electron microscopic approaches are not only sensitive and direct but they often decisively complement the other approaches.

II. General Utility

Hybridization techniques have become fashionable, and they are sometimes used when it is unnecessary. When should these techniques be used? What is required for their use? The prime application of these techniques is for the detection of viral genomes that cannot be disclosed by tests for infectivity. In principle, there is no more sensitive technique than simple determination of infectivity. However, if the viral genome is defective and unable to replicate, if the viral genome is integrated in whole or in part into the cellular chromosome, or if the viral genome is present as an episome, then hybridization techniques are indispensible. Systems, especially in the RNA tumor virus field which require helper viruses for expression of infectivity, also call for nucleic hybridization technology. *In vitro* systems for synthesis of viral DNA or RNA at some stage require identification and quantitation of the products by specific hybridization. Nucleic acid hybridization provides the only accessible method for the identification of messenger RNA. These methods are particularly valuable for decisive identification

of viruses or viral genetic material. They provide the ultimate methods for the determination of homology between viruses both in kind and in degree. They make it possible to determine the percentage of the genome or transcripts of the genome present in cells or tissue. The technique of cytohybridization actually makes possible localization of viral genetic material to specific cell type and even intracellular site. For sensitivity and quantitation of nucleic acids hybridization approaches are the methods nonpareil.

There are, however, requirements that must be met before these techniques can be employed. First, the identity of the nucleic acid being sought must be known or suspected. There is no way that one can detect a specific genome in whole or in part without the use of the appropriate viral probe unless through an accident of partial homology. Second, the nucleic acid used as probe must be purified and radiolabeled to a high degree of specific activity. The general limitation on the minimum amount of DNA that can be detected in liquid systems at present depends upon the complexity of the genome. However, it is already within the realm of possibility to improve upon this level of sensitivity up to 10-fold. This can be done by using probes of restricted fragments of nucleic acid that might be present in the cell system under analysis. Cytohybridization is a less sensitive technique at present, but it is advantageous for certain purposes. The overriding power of these techniques is the certainty with which it is possible to uncover the presence of specific nucleotide sequences. Viral genetic material that defies detection by any other approach can be defined with precision by the proper use of the technology we are about to describe.

III. Basic Methods

A. PREPARATION AND RADIOLABELING OF PROBES

The purity and specific radioactivity of the nucleic acids used as probes are crucial to the conduct of successful nucleic acid hybridization. The representative nature or fidelity of the probe if copied sequences are being used is also important. In some cases a suitable probe can be achieved by direct incorporation of radiolabeled uridine or thymidine during replication of the virus in cell culture. However, with many viruses there is insufficient incorporation. In general nucleic acids with specific radioactivity in excess of 10^6 cpm/μg, preferably about 10^7 cpm/μg, are needed. If labeling *in vivo* does not reach this level of radioactivity, then labeling during *in vitro* synthetic reactions is needed. None of the current *in vitro* methods of labeling yields completely representative probes; label may be unevenly distributed in the repair synthesis methods, or some sequences may not be copied into the probe.

These shortcomings do not obviate the value of such probes for most purposes, but they do make it mandatory to carry out appropriate reconstruction or saturation analyses. Methods of labeling nucleic acids by direct or indirect iodination should in principle avoid the deficiencies of the other methods in the construction of representative probes. However, iodination introduces its own problems; to date this approach has not been as useful as had been hoped.

In general, if labeling of nucleic acids can be accomplished in infected cell cultures, then this is the preferable method. However, labeling of DNA *in vitro* by repair synthesis is a versatile technique that gives most satisfactory results. In situations in which the nucleic acid that serves as template is available only in minute amounts, the synthesis of complementary nucleic acids, either DNA or RNA, can provide excellent probes and at the same time augment the amount available.

The purity of the nucleic acid used as probe is the most critical element in specific hybridization. Virus harvested from extracellular fluids rather than from infected cells is the better source of probe nucleic acid, particularly if methods involving complementary RNA (cRNA) or complementary DNA (cDNA) are employed. Virus harvested from cells carries with it contaminating cellular DNA. Even virus from extracellular fluids is contaminated with host–cell DNA which is not always eliminated by DNase treatment because of protection by extraneous proteins. The first step, then, is to procure virus which is highly purified by any method that conserves the integrity of the virion so that treatment with DNase will not cause fragmentation of the encapsidated genome. Next, the extracted viral DNA should be rigorously purified. The genome should emerge from purification largely unfragmented so that it can be separated from cellular nucleic acids on the basis of physical properties such as size, as well as density or supercoiled state. Physical homogenity is the aim. Analysis of the isolated components of nucleic acid both by optical and radioisotope counting methods is desirable since contaminating cellular DNA may not have been labeled. The final test of purity of the probe is a complete set of analyses of the behavior of the probe during hybridization in the presence of heterologous as well as homologous nucleic acids. These tests should include DNA from uninfected host–cell material. There is always some degree of nonspecific background hybridization, but the level should be low, and it should be consistent for each probe prepared.

The probes include DNA radiolabeled either *in vivo* or *in vitro*, cRNA, and cDNA. The isotopes of most utility are ^{3}H, ^{32}P, and ^{125}I. Methods of iodination are discussed last in this section.

1. *DNA Labeled in Vivo*

Some viral DNAs can be labeled to high specific activity during replication in cell cultures, but other viral DNAs incorporate radiolabeled thymidine much less efficiently. Viruses such as herpes simplex which induce virus-specific thymidine kinase can be labeled to relatively high specific activities, as shown by Davis and Kingsbury (1976). In contrast, human cytomegaloviruses (CMV) which appear not to induce novel thymidine kinases (J. Estes and E.-S. Huang, unpublished data) cannot be labeled well *in vivo*. The efficiency of incorporation of radiolabeled thymidine into viral DNA works best if viral DNA is replicated through salvage pathways rather than by *de novo* synthesis. In the case of Epstein–Barr virus, (EBV), incorporation of tritiated thymidine into viral DNA is also inefficient, and it is difficult to obtain EBV DNA with specific radioactivities of more than 10^5 cpm/μg. This is generally inadequate for hybridization techniques. With this virus the situation is complicated further by the very low yields of virus per unit of culture fluid, and it is impractical to add enough radioisotope to the large volumes of culture medium needed to produce sufficient virus. If, however, viral DNA can be radiolabeled *in vivo*, then this is usually considered the method of choice because of the uniform radiolabeling that results.

Herpes simplex virus (HSV) types 1 and 2 induce their own specific thymidine kinase in virus-infected cells. The salvage pathway as well as *de novo* viral DNA synthesis is utilized in the presence of viral replication. Tritiated thymidine of high specific activity (60 Ci/m*M*) in aqueous sterile H_2O is added to HSV type 1-infected cells at a concentration of 50 μCi to 100 μCi per milliliter immediately after virus adsorption. The most economical and efficient procedure is to grow the cells in Wheaton roller bottles; 10–15 milliliters of culture media is enough to cover a monolayer of 5×10^7–1×10^8 cells in the bottle. Twenty-four hours after infection HSV can be purified from the cytoplasmic fraction of the infected cells; 48–72 hours after infection the virus can be purified from extracellular fluid. The [^{3}H] HSV DNA is purified from virions, as previously described (Huang *et al.*, 1973). The specific activities obtained for HSV 1 and HSV 2 are $1–2 \times 10^7$ and $2–5 \times 10^6$ cpm/μg; respectively (Davis and Kingsbury, 1976; E.-S. Huang, unpublished data).

2. *DNA Labeled in Vitro*

The most widely used method for labeling of viral DNA *in vitro* is nick translation or repair synthesis with DNA polymerase I (Aposhian and Kornberg, 1962). In this technique, purified viral DNA is first nicked under carefully controlled conditions with pancreatic DNase I and then subjected to repair synthesis in the presence of radiolabeled [^{3}H]TTP or [^{125}I]dCTP (Shaw *et al.*, 1975). DNase digestion has to be done carefully so as to avoid undue hydrolysis. The nicks introduced are assumed to be distributed at random through the genome. Since repair synthesis is initiated at the nicks, the introduction of radiolabel should also be at random sites along the genome. Under optimal conditions, up to 35% of ^{32}P murine CMV genome

can be replaced in either strand by newly synthesized [^{3}H]DNA (E.-S. Huang, unpublished results). The conditions, especially the temperature in which repair synthesis is conducted, are crucial in order to prevent redundant and branched replication in which the newly synthesized strand segments are themselves copied. Such structures behave anomalously during digestion with S1 single-strand specific nuclease. In any case, viral DNA with specific activities between 10^6–10^7 cpm/μg can be produced with this method (Nonoyama and Pagano, 1972; Frenkel *et al.*, 1972; Huang *et al.*, 1973).

DNA polymerase I is used to prepare tritium-labeled viral DNA *in vitro*, and was purified according to Jovin *et al.*, (1969), as described previously (Huang *et al.*, 1973).

The details of the *in vitro* synthesis reaction were originally described by Nonoyama and Pagano (1972). Two micrograms of purified EBV DNA are incubated with 5×10^{-2} μg of DNase I for 10–20 minutes at 37 °C in 0.45 ml of 70 m*M* potassium phosphate, pH 7.4, 1 m*M* 2-mercaptoethanol, and 7 m*M* $MgCl_2$. DNase is inactivated by heating the mixture for 10 minutes at 70 °C. The nicked DNA is then labeled in a 0.5 ml reaction which contains: 70 m*M* potassium phosphate, pH 7.4, 1 m*M* 2-mercaptoethanol, 7 m*M* $MgCl_2$, 0.1 m*M* each of dATP, dCTP, and dGTP, 250 μCi of [^{3}H] TTP (20 Ci/m*M*), and 2 units of DNA polymerase I. The mixture is incubated at 18 °C until incorporation of [^{3}H] TMP (measured by counting TCA precipitates of aliquots taken during the course of the reaction) has reached a plateau (approximately 5 hours); then Sarkosyl and neutralized EDTA are added to 1% and 10 m*M*, respectively, and the mixture is passed through G-50 Sephadex equilibrated with 10 m*M* Tris-HCl, pH 7.4, neutralized 1 m*M* EDTA, and 0.1% Sarkosyl. The TCA-precipitable radioactivity in the fractions eluted from the column are pooled and mixed with an equal volume of water-saturated phenol. Following centrifugation, the DNA in the aqueous phase is precipitated overnight with salt-saturated ethanol at −20 °C. The specific activity of the DNA is estimated before G-50 Sephadex chromatography from the DNA concentration and the TCA-precipitable radioactivity in an aliquot of the reaction mixture.

3. *Messenger RNA*

Viral mRNA can be labeled in infected cells at various times after infection with [^{3}H]uridine or ortho ^{32}P or *in vitro* by direct iodination. The specific activity of mRNA is dependent on the amount of radioactive material used. In general, for short-term labeling in tissue culture, larger quantities of isotope (up to 100 μCi/ml) can be used; for long-term labeling the quantity should be decreased to 3–10 μg/ml in order to avoid radiation damage. The times selected for labeling depend on the purpose of the experiment.

Messenger RNA of high specific activity can be obtained by iodination

of mRNA after purification with ^{125}I by the thallium chloride technique. However, the labeled product is suitable only for hybridization purposes and not for size characterization; this is due to fragmentation of the iodinated product. Specific activities of viral mRNA up to $5–10 \times 10^7$ or even higher can be obtained, but undesired effects are encountered with specific activities greater than 5×10^8 cpm/μg. The ideal specific activity for consistent results and low background is between 2×10^7 and 1×10^8 cpm/μg (our unpublished results). The method for iodinating viral mRNA is described in Section III, A,6.

a. Preparation of Viral mRNA. Several features affecting extraction of mRNA, both viral and cellular, should be mentioned. (i) Most but not all cellular and viral mRNA contain poly(A) stretches at the 3′OH end of the RNA molecule (Edmonds and Caramela, 1969; Edmonds and Kopp, 1971; Sheldon *et al.*, 1972; Hadjivassilions and Brawerman, 1966; Kates, 1970; Phillipson *et al.*, 1971). At neutral pH and cold temperatures, the poly(A)-containing mRNA is retained in the nonaqueous phase during phenol extraction (Georgiev and Mantieva, 1962) from which it can be recovered by reextraction with a slightly alkaline buffer (pH 9) (Brawerman *et al.*, 1963). (ii) Retention of poly(A)-containing RNA in the nonaqueous phase at neutral pH is caused by interaction between the poly(A) and the denatured ribosomal proteins. Tris is protonated at neutral pH; a solution of 0.1 *M* Tris has a concentration of monovalent cations equal to 0.1 *M* K^+. This monovalent cation will promote the interaction of poly(A) and protein. This kind of interaction can be overcome by extraction at slightly alkaline pH; at pH 9 Tris is in an almost nonionized condition (Edmonds and Caramela, 1969). (iii) Phenol extraction at alkaline pH is very effective for mRNA extraction, but at low temperature DNA is extracted together with mRNA. Although the cell DNA can be removed by digestion with pancreatic DNase, contamination of the DNase preparation with RNase causes fragmentation of the RNA; consequentially there are almost always problems in recovery of mRNA. The hot phenol–SDS method (Girard, 1967) for mRNA extraction facilitates recovery of mRNA; this method is as efficient as phenol extraction at pH 9, and it does not trap mRNA in the nonaqueous phase. Also DNA is excluded by retention in the nonaqueous phase.

Extraction of polysomal RNA or RNA from the cytoplasmic fraction is best carried out in an ice bath. The sample in 0.05–0.1 *M* Tris-HCl, pH 9, and 0.5% SDS is extracted three times with equal volumes of water-saturated redistilled phenol (80% phenol). The mixture of sample and phenol is vigorously stirred for 5 minutes and then centrifuged at 5,000 rpm in the Sorvall HB-4 or SS-34 rotor (10,000–12,000 *g*) for 5 minutes. The upper aqueous phase containing mRNA and rRNA is saved. The nonaqueous phase (phenol phase and interphase) is reextracted with a suitable volume

of 0.1 *M* Tris-HCl, pH 9, and 0.5% SDS to recover the RNA trapped in the nonaqueous phase. The aqueous supernatant fractions of each extract are pooled, and RNA is precipitated with 2.5 volumes of ethyl alcohol in the presence of 0.1 *M* NaCl at −20°C overnight. The precipitate is collected by centrifugation in the HB-4 or SS-34 rotor at 10,000 rpm for 30 minutes and washed twice with 70–90% alcohol containing 0.1 *M* NaCl to remove residual phenol. The precipitate can be dissolved in any desired buffer and stored at −20°C, but preferably at −70°C.

For the extraction of RNA from nuclei or whole cells, the hot phenol–SDS method is recommended to avoid entrapment of RNA in cellular DNA (Girard, 1967; Brawerman, 1974). The extraction procedures are identical to those used for polysomal or cytoplasmic RNA except that at least the first cycle of SDS–phenol extraction is carried out in a 60°C water bath. After vigorous extraction at 60°C for 5 minutes, the sample is chilled quickly in an ice bath. Under these conditions RNA is released to the aqueous phase while cell DNA remains in the nonaqueous phenol phase. Separation of aqueous and nonaqueous phases by centrifugation and alcohol precipitation follow.

b. Preparation of Poly(A)-Containing mRNA. Poly(A)-containing mRNA has several properties useful for extraction. (i) Due to complementary base pairing it can absorb to poly(U)-Sepharose or oligo(dT) cellulose columns at high ionic strengths. (ii) The poly(A) segment of mRNA is able to bind to nitrocellulose filters in a manner similar to denatured single-stranded DNA in the presence of 0.5 *M* KCl and low levels of actinomycin; the size of the poly(A) stretch should be greater than 50 nucleotides (Lee *et al.*, 1971). This RNA does not adsorb to filters in the presence of detergent, and it can be removed from nitrocellulose filters with 0.5% SDS in 0.1 *M* Tris-HCl, pH 9. (iii) Poly(A) also binds to denatured protein in the presence of sufficiently high concentrations of monovalent cations (Brawerman, 1974).

Based on these properties, mRNA containing poly(A) can be isolated by poly(U)-Sepharose, poly(U) glass filters and oligo(dT) cellulose, and by B-6 Millipore filters.

Poly(U)-Sepharose (Adesnik *et al.*, 1972) and oligo(dT)-cellulose (Aviv and Leder, 1972) are commercially available. Poly(U) glass filters can be prepared by coupling poly(U) to glass fiber filters by means of UV irradiation. The method has been described (Sheldon *et al.*, 1972; Adesnik *et al.*, 1972). In brief, 0.1 ml of poly(U) stock solution (2 mg/ml sterilized distilled water) is applied to Whatman GF/C filters. After drying at room temperature, the filters are dried in a vacuum at 37°C and then UV-irradiated on both sides for 5 minutes with a 15–30 W GE germicidal lamp (distance, 20 cm). The unadsorbed poly(U) is rinsed away with sterile distilled water. The filter is dried at 37°C and kept at 4°C.

Before hybridization of mRNA to the poly(U) glass filters, the filters are first washed with distilled water by Millipore filtration in individual holders. The number of filters used is totally dependent on the amount of RNA expected; several layers of filters can be used. The RNA solution without DNase digestion in SDS buffer (1% SDS, 0.001 *M* EDTA, 0.1 *M* NaCl, 0.01 *M* Tris-HCl, pH 7.4) is slowly passed through the poly(U) glass filter at a flow rate of 1–2 ml per minute at room temperature. After washing with SDS buffer, the poly(U)-containing mRNA is eluted from the filter with low ionic strength 0.1 × SDS buffer. The elution can be done by crushing the filter in 0.01 × SDS buffer and removing the glass fiber by centrifugation. The poly (A)-containing RNA is precipitated by alcohol.

The method for isolation of poly(A)-containing mRNA by poly(U)-Sepharose 4B is described in detail in the Pharmacia poly(U)-Sepharose 4B Bulletin. In brief, the gel is first swollen in 1 *M* NaCl (in 0.05 *M* Tris-HCl, pH 7.5) for 5 minutes and washed with 0.1 *M* NaCl in 0.05 *M* Tris-HCl, pH 7.4. The column is then equilibrated with a concentrated salt buffer (CSB; 0.7 *M* NaCl, 0.05 *M* Tris-HCl, 0.01 *M* EDTA, and 25% formamide, final pH 7.5). The RNA sample in detergent solution (1% lauroyl-sarcosine, 0.03 *M* EDTA) is diluted 5 times with CSB and applied to the column. After sample application the column is washed with CSB buffer, and the poly(A)-containing mRNA is recovered with eluting buffer(EB; 0.01 *M* potassium phosphate, 0.01 *M* EDTA, 0.2% lauroyl-sarcosine, 90% formamide, final pH 7.5).

The procedure for isolation of poly(A)-containing mRNA with oligo(dT) cellulose columns is described by Aviv and Leder (1972). The RNA solution is applied to the oligo(dT) column in the presence of 0.5 *M* KCl (in 0.01 *M* Tris-HCl, pH 7.5), and the mRNA is eluted from the column with 0.01 *M* Tris-HCl, pH 7.5.

Although up to 60 μg of poly(A)-containing RNA can adsorb to a single nitrocellulose Millipore filter in the presence of 0.5 *M* KCl, the technique in which Millipore filters are employed is not popular (Brawerman *et al.*, 1972). This is due to extensive contamination of isolated mRNA with rRNA. The detailed procedure is described by Brawerman *et al.* (1972). The RNA in KCl buffer (0.5 *M* KCl, 1 m*M* $MgCl_2$, 0.01 *M* Tris-HCl, pH 7.6) free of detergent and at a concentration less than 300 μg/ml (Brawerman, 1974) is slowly passed through a Millipore nitrocellulose filter (0.5 ml or less per minute). The filter is washed with KCl buffer and then kept in 0.5–1.0 ml of elution buffer (containing 0.5% SDS, 0.1 *M* Tris-HCl, pH 9) in ice for about 30 minutes to elute the poly(A)-containing mRNA from the filters.

4. *Complementary RNA: Practical Theory*

In cases in which very small amounts of nucleic acid are available to serve as probes or the specific radioactivity achieved by direct incorporation of

label into the probe nucleic acid is too low, then the use of cRNA may be of great value. If cellular DNA that contains hybridizable sequences is denatured and affixed to membrane filters, then self-annealing of DNA is prevented. Instead the DNA is accessible for hybridization with complementary sequences in the probe nucleic acid, in this case, cRNA. Complementary RNA, preferably in excess, is applied to duplicate filters containing the immobilized DNA to be tested and hybridized under appropriate ionic conditions (0.3–0.9 *M* NaCl) at 66°C for a prolonged period, such as 16–20 hours. Usually about 20–50 μg of DNA are affixed to each filter; this quantity is confirmed by a diphenylamine reaction after hybridization has been carried out. In the neighborhood of 100,000 counts of the cRNA (specific activity, 1×10^7 cpm/μg) are usually applied to each filter. Extensive rinsing and treatment with RNase are important steps in the procedure to eliminate nonspecifically adherent cRNA. RNA–DNA hybrid duplexes are resistant to RNase. Hybridization of cRNA to homologous sequences of DNA is directly proportional to the concentration of homologous DNA until all of the sequences present in the probe and in the homologous DNA have been hybridized, in which case saturation conditions are met.

Procedure. This procedure was used for the preparation of EBV cRNA (Nonoyama and Pagano, 1971). Highly purified *Escherichia coli* DNA-dependent RNA polymerase was prepared according to Burgess (1969). The reaction mixture contained 0.04 *M* Tris, pH 7.9, 0.01 *M* $MgCl_2$, 0.1 m*M* dithiothreitol, 0.15 *M* KCl, 0.15 mg/ml bovine serum albumin, 0.15 m*M* GTP, ATP, and CTP, 0.15 m*M* [^{3}H]UTP (17–30 Ci/m*M*), 16 units of enzyme (400 units per milligram protein) and 2 μg EBV DNA in 0.25 ml. The mixture was kept at 37°C for 2–5 hours when the reaction reached a plateau, the DNA was then digested with DNase (RNase-free batch, Worthington Biochemical, Freehold, N.J.) at the concentration of 20 μg/ml for 20 minutes at 37°C. Yeast RNA (1 mg) and SDS (0.5% final concentration) were added, and the mixture was chromatographed through a Sephadex G-50 column (1.1 × 30 cm) in Tris-EDTA (TE; 0.05 *M* Tris-HCl, pH 7.4; 0.001 *M* EDTA) with 0.1% SDS. The first peak was collected, treated with water-saturated phenol, and dialyzed against TE with 0.01% SDS at 4°C for 36 hours with three changes of the buffer. The incorporation efficiency in these conditions was 3 to 6% of the added [^{3}H]UTP. From the specific radioactivity of the [^{3}H]UTP and the guanine-cytosine content of EBV DNA, the product was expected to have 10^7 cpm/μg.

When the synthesized cRNA was analyzed by velocity sedimentation on sucrose gradients, radioactivity was distributed from 16 S to 4 S, with an obvious peak at the 12 S to 16 S region. As the incubation time was so long, some degradation of the RNA could be expected by a trace of RNase in the reaction mixture.

5. *In Vitro Synthesis of DNA Transcripts (cDNA) of RNA Virus Genomes*

The largest components of RNA tumor virus genomes are 60–70 S RNA (Gillespie *et al.*, 1975; Robinson *et al.*, 1965). At an early stage of investigation, there were indications that fragmentation of the viral genomes occurred upon heat treatment; the fragments produced are heterogeneous in size (Duesberg, 1970). In the case of avian tumor viruses, this 60–70 S RNA consists predominantly of two 35 S RNA subunits with approximate molecular weights of 3×10^6 (Duesberg and Vogt, 1973). These data imply that the 60–70 S DNA is diploid and composed of two identical subunits.

Transcription of RNA tumor virus genomes *in vitro* by viral RNA-dependent DNA polymerase generates chiefly short DNA transcripts ranging up to 2×10^5 MW in size (Rokutanda *et al.*, 1970; Temin and Baltimore, 1972; Duesberg and Canaani, 1970). Duesberg and Canaani demonstrated that DNA made *in vitro* with Rous sarcoma virus (RSV) renders most of viral RNA resistant to RNase digestion; Duesberg and Canaani (1970) and Garapin *et al.* (1973) suggested that the synthesized DNA is complementary to the entire viral genome.

Recently the difficulty in obtaining full-length viral DNA transcripts *in vitro* has been overcome by the finding of the optimal triton X-100 concentration necessary for expression of viral DNA synthesis. With optimal triton concentration (0.0225%, v/v) about 1–10% of the transcribed DNA has a size between $2.5–3.7 \times 10^6$ MW which is complementary to the total viral RNA (Junghaus *et al.*, 1975).

Radioactive DNA transcripts of an RNA tumor virus were used by Gelb *et al.* (1971) to detect the viral DNA in mammalian cells in a murine leukemia virus system (MuLV). The virus DNA was made in reaction mixtures containing 0.05 *M* Tris-HCl, pH 7.8, 0.002 *M* dithiothreitol, 0.06 *M* NaCl, 5×10^{-4} *M* deoxyribonucleotide triphosphate (specific activity of dXTP was 161 mCi/m*M*), 0.014% Triton X-100, and 100 μg of virus protein per milliliter. The reaction mixture was incubated at 37°C for 18 hours. The double-stranded DNA was then extracted by a phenol–SDS method, and the viral RNA was digested by pancreatic RNase at 10 μg/ml for 2 hours at room temperature. The details are given in Gelb *et al.* (1971). The specific activity of ^{3}H-labeled murine leukemia virus DNA was around 2.5×10^5 cpm/μg. Reassociation kinetics analysis of this *in vitro* [^{3}H]MuLV DNA showed a Cot_{50} of $2.8–3.4 \times 10^3$ mol sec/liter, equivalent to a genome complexity (molecular weight) of $5.0–6.1 \times 10^6$ (Gelb *et al.*, 1971).

The procedure and the reaction mixture used by Junghaus *et al.* (1975) for *in vitro* synthesis of full-length DNA transcripts of RSV RNA are briefly described for reference. Rous sarcoma virus equivalent to 1.25 mg of viral protein was added to a reaction mixture containing 10^{-4} *M* dATP, dGTP;

10^{-5} *M* dTTP, dCTP; 0.002 *M* magnesium acetate, 0.03 *M* dithiothreitol; 0.1 *M* Tris-HCl, pH 7.6; 0.825 μM [^{3}H]TTP (20 Ci/m*M*); and 0.0225% TritonX-100. The total volume of the reaction mixture was 4 ml. The reaction was carried out at 41°C for 18 hours and was terminated by the addition of EDTA to 0.015 *M*, SDS to 1%, NaCl to 0.1 *M*, and β-mercaptoethanol to 2%; 80 μg of denatured salmon sperm DNA was added as carrier. The product DNA was extracted with phenol, and viral RNA was removed by digestion with pancreatic RNase (Gelb *et al.*, 1971). The specific activity of the viral DNA synthesized was around 2×10^5 cpm/μg.

6. *Iodination Methods: Direct and Indirect Labeling of DNA and RNA*

There are several methods for labeling of nucleic acids *in vitro*. These include (i) tritium exchange (Borenfreund *et al.*, 1959), (ii) methylation of DNA by tritium-labeled dimethyl sulfate (Smith *et al.*, 1967), and (iii) iodination of nucleic acids in the presence of thallic chloride (Commerford, 1971). The specific activity achieved by tritium exchange and methylation does not meet the requirements for a sensitive probe in either membrane hybridization or reassociation kinetics analysis.

The technique for direct labeling of nucleic acid with radioactive iodine in the presence of thallic chloride at low pH was first established by Commerford (1971). The method yields nucleic acid of very high specific activity and offers economical and simple processing. The ^{125}I is incorporated into DNA through a covalent bond as 5-iodocytosine (Commerford, 1971). There are two ways to label DNA or RNA with ^{125}I: directly and indirectly. The direct iodination technique has recently been modified for labeling of 5 S RNA, mRNA, or denatured DNA (Prensky *et al.*, 1973; Tereba and McCarthy, 1973; Scherberg and Refetoff, 1974). The technique for labeling DNA and RNA is essentially the same except that the DNA should be maintained in the single-stranded state and should be kept from self-annealing during iodination.

Several important considerations should be kept in mind for high efficiency of labeling. (i) The specific activity of the nucleic acid is dependent on the amount and molar ratio of iodine to cytosine used in the iodination system. For reproducible specific activities, a constant molar ratio of iodine to cytosine should be used (optimal ratio about 1). (ii) The purity of thallium chloride (K & K Chemical Co., Plainview, N. Y.) plays an important role in the efficiency of iodination. Poor iodination will result if a chelating agent is present; the amount of thallium ion should be in excess of any chelating agent present. A molar ratio of $TlCl_3$ to iodine of 6 to 1 is popularly used (Commerford, 1971). A fresh preparation of thallium chloride solution at pH 5 is recommended. (iii) The pH of the reaction mixture is critical; it should be 4.5–5.0. (iv) The efficiency of iodination of double-stranded DNA is low. The DNA should be denatured first by boiling in low ionic strength buffer or distilled water. The ionic strength of the iodination buffer should be maintained below 0.1 *M* Na^+ in order to prevent reannealing at the iodination step.

An example of a procedure for labeling human CMV DNA is as follows: Human CMV DNA is dialyzed against distilled water, denatured by boiling for 10 minutes, and rapidly chilled in an ice bath. The DNA solution is then adjusted to pH by adding sodium acetate–acetic acid buffer (final concentration, 0.01 *M* sodium acetate). For iodination of single-stranded DNA or RNA, when the denaturation step is not needed, the sample is simply subjected to dialysis against 0.1 *M* acetate buffer, pH 5. The reaction mixture (Commerford, 1971), assembled at 0°C, contains in a total volume of 100 μl, 0.1 *M* (or 0.01 *M* for denatured DNA) acetate buffer (pH 5); 2 μg denatured CMV DNA; 0.25 m*M* KI in chemical equilibrium with the desired amount of ^{125}I, and 1.5 m*M* $TlCl_3$ (added last in sodium acetate buffer, pH 5). The reaction is mixed and heated to 60°C in a water bath for 15 minutes and then chilled in ice. The specific activity can be controlled by the length of the reaction time. Twenty-five microliters of freshly prepared 0.1 *M* Na_2SO_3 are added to reduce the excess amount of thallium chloride; β-mercaptoethanol at a final concentration of 30 m*M* is recommended in place of Na_2SO_3. The noncovalently bound ^{125}I can be removed by competition with cold sodium iodide in order to reduce the nonspecific background radioactivity after this stage. The pH of the reaction mixture is then raised to pH 9 by adding 0.05 ml of 1 *M* ammonium acetate–0.5 *M* ammonium hydroxide solution. The mixture is then heated at 60°C for 15 minutes to disassociate the unstable intermediate products (Commerford, 1971). At the end of the reaction the mixture is chromatographed on a Sephadex G-50 or hydroxyapatite column to recover the DNA or RNA (for details, see Commerford, 1971; Prensky *et al.*, 1973).

Iodination of nucleic acid by the direct method incurs treatment with oxidizing and reducing reagents at high temperature and at high and low pH. Also, native DNA cannot be labeled efficiently. Indirect methods of labeling viral DNA and cRNA are available that avoid exposure of DNA to high temperature, unfavorable pH, and oxidizing or reducing conditions. DNA can be recovered in the native state after iodination. The indirect method involves first synthesis of 5-^{125}iodo-dCTP or 5-^{125}iodo-CTP and then use of these reagents as precursors for repair synthesis of viral DNA *in vitro* and synthesis of cRNA.

The method for iodination of dCTP and CTP is essentially the same as the method described above for iodinating viral DNA and RNA, except that after iodination the [^{125}I]dCTP and CTP are recovered by DEAE-cellulose chromatography. The iodination reaction mixture is diluted to a

salt concentration of less than 0.01 *M* and loaded on a DEAE-cellulose column (Whatman DE52, 0.9 by 22 cm) that has been prewashed with 0.3 column volume of 1 *M* triethylamine carbonate, pH 8.0, and pre-equilibrated with 0.01 *M* triethylamine carbonate, pH 8. The triethylamine carbonate is prepared by passing CO_2 into a mixture of 170 ml water and 30 ml redistilled triethylamine in ice until a single phase of triethylamine carbonate forms. The pH of the solution is adjusted by adding CO_2 to pH 8. After loading, the column is washed with 0.01 *M* triethylamine carbonate, and then the sample is eluted with a linear gradient (0.01–0.5 *M*) of triethylamine carbonate, pH 8. The peak fractions containing 5-iodo-dCTP or 5-iodo-CTP are pooled and lyophilized to dryness. The final product is dissolved in H_2O and stored at −20 °C.

Synthesis of ^{125}I-labeled viral DNA or cRNA are essentially as described for ^{3}H viral DNA and [^{3}H]cRNA earlier (Sections III,A,2 and 4). The details are given by Shaw *et al.* (1975).

B. DNA–DNA Hybridization on Nitrocellulose Filters

The classic method of hybridization is with probe DNA to denatured DNA affixed to membrane filters. The DNA probe can be radiolabeled either *in vivo* or *in vitro*. This method represents a direct approach to hybridization, but it presents a number of problems that have curtailed its use with the availability of new methods. Direct DNA–DNA hybridization requires relatively large amounts of probe DNA in contrast to kinetic methods of hybridization which are conducted in liquid media (Section III,F). As noted above, the difficulty of attaining sufficiently high specific radioactivity can be overcome with radiolabeling of the DNA *in vitro*. The amounts usually required for repeated hybridization tests mean that the labeled DNA must be prepared more frequently than if it is to be used in other hybridization systems. Another problem with DNA–DNA hybridization is self-annealing of the DNA probe. Ideally, DNA–DNA hybridization utilizes separated complementary strands as probe. These in turn have special uses and shortcomings. Also, it is not as easy to eliminate unhybridized DNA as cRNA; in cRNA–DNA hybridization RNase can be used since the RNA in hybrid molecules is not digested by the enzyme. Nevertheless, DNA–DNA hybridization has given important leads (Fujinaga and Green, 1966; zur Hausen and Schulte-Holthausen, 1970) and will probably continue to prove useful in the future. The method for DNA–DNA hybridization is similar to RNA–DNA hybridization except that in DNA–DNA hybridization S1 or *Neurospora crassa* single-stranded nuclease is used instead of RNase to digest nonhybridized single-stranded DNA.

C. RNA–DNA Hybridization on Nitrocellulose Filters

The principles and procedures of RNA–DNA hybridization were described by Gillespie and Spiegelman (1965). The denatured DNA is first immobilized on nitrocellulose filters and then annealed with radioactive virus-specific RNA. There are two ways to denature the DNA for immobilization: one is by boiling in low salt solution and rapid chilling, and the other is by denaturation in alkali. An example of membrane-filter hybridization is shown in Fig. 1.

Fifty micrograms of HEp-2 DNA with graded amounts of CMV DNA in 2 ml of 0.1 × SSC in 1 m*M* EDTA were denatured in 0.5 *N* NaOH for 2 hours at 37 °C. The DNA solution was neutralized with 1.1 *N* HCl in 0.2 *M* Tris in an ice bath and then adjusted to 6 × SSC. The solution was slowly passed through a Bact-T-flex type 6 (Schleicher and Schuell Co., Keene, N. H.) nitrocellulose membrane filter (prewashed and soaked in 6 × SSC). After filtration washing, the filters were dried at room temperature for at least 3 hours or overnight and baked in a vacuum oven at 80 °C for 3 hours. The filters can be stored in the refrigerator or at room temperature for several months without loss of hybridization capacity.

The DNA filters were then immersed in 1 ml of 6 × SSC containing CMV [^{3}H]cRNA (1.5×10^3 cpm; specific activity, 1×10^7 cpm/μg), 1 mg of yeast RNA, and 0.1% SDS. The hybridization was carried out at 66 °C (20°–25 °C below T_m) for 20 hours. Hybridization can be conducted at lower temperature (40 °C) in the presence of 50% formamide for 40 hours; the reaction mixture contained 1.5×10^5 cpm [^{3}H]CMV cRNA, 1 mg yeast RNA in 50% formamide, 0.9 *M* NaCl, 0.1% SDS, and 0.01 *M* Tris-HCl, pH 7.9. After hybridization the filters were extensively washed with 2 × SSC. The unhybridized [^{3}H]cRNA was removed by digestion with 40 μg/ml of RNase (preheated at 80 °C for 10 minutes to inactivate contaminating DNase) at 37 °C for 30 minutes. The filters were then washed by filtration on both sides with 2 × SSC, dried, and counted.

1. *Reconstruction Curve*

Figure 1A and B show reconstruction curves of CMV and EBV DNA hybridization, respectively. These data provide the basis for calculation of the number of viral genome equivalents detected by hybridization. These curves were constructed from the amount of radioactive CMV cRNA or EBV cRNA that hybridized to graduated amounts of CMV DNA or EBV DNA in the presence of 50 μg of HEp-2 or calf thymus DNA. If the molecular weight of CMV DNA and diploid human cell DNA are 10^8 and 4×10^{12}, respectively, then 0.1 μg of CMV DNA in 50 μg of human tissue DNA

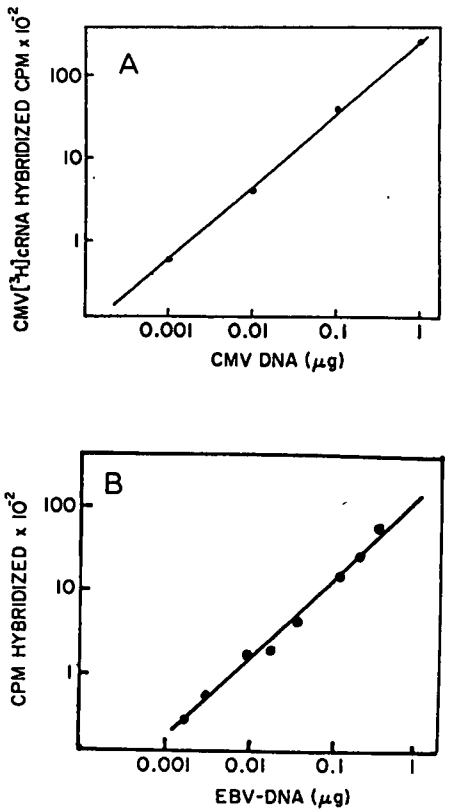

FIG. 1. (A) Hybridization of CMV cRNA to CMV DNA. Graded amounts of CMV DNA were mixed with 50 μg of HEp-2 DNA and denatured in 0.5 *N* NaOH for 2 hours at 37°C. The DNA solution was neutralized with 1.1 *N* HCl in 0.2 *M* Tris, adjusted to 6 × SSC, and immobilized on filters. The input cRNA for each filter was 1.5 × 10^3 cpm (specific activity was about 10^7 cpm/μg). The amount of DNA retained on the filter, determined by a diphenylamine test after hybridization, remained constant. The background hybridized counts for 50 μg of HEp-2 (130 cpm) were subtracted from each value. (From Huang *et al.*, 1973). (B) Calculation of numbers of EBV genomes. EBV DNA in the quantities indicated was mixed with 50 μg HeLa cell DNA and denatured in alkali. DNA–RNA hybridization was carried out according to Gillespie and Spiegelman (1965) except that SDS was present at the final concentration of 0.1% during hybridization. The DNA concentrations on the filters were determined by the diphenylamine test after hybridization, and the hybridized counts were expressed per 50 μg DNA. The input cRNA was 75,000 cpm per filter. The hybridized value for 50 μg of HeLa DNA alone was subtracted. (From Nonoyama and Pagano, 1971).

TABLE I
DNA–RNA HYBRIDIZATION TESTS[a,b]

DNA on filter	cRNA hybridized (cpm/50 μg DNA)	Estimated number of genome equivalents per cell
HR-1 Burkitt lymphoma	12,131	680
32°C for 10 days	17,392	810
IF negative[c]	596	32
Raji Burkitt lymphoma		
IF negative	1126	65
Chromosomes[d]	1099	62
EBV infected[e]	22,617	1170
6410 myelogenous leukemia[f]	803	45
F-265 normal patient[g]	1650	100
NC-37 human healthy donor[g]	1343	80
HeLa human carcinoma	152	< 2
HEp-2 human carcinoma	126	< 2

[a] From Nonoyama and Pagano (1971).
[b] The conditions for hybridization were the same as in Fig. 1B. All DNA was prepared by treatment with SDS and Pronase followed by phenol extraction; 150 cpm, the hybridized value for HeLa cell DNA, was subtracted to estimate viral genome number equivalents.
[c] HR-1 cell line that no longer sheds EBV.
[d] Raji cells were arrested at metaphase by 0.05 μg/ml of Colcemid, and chromosomes were prepared according to Maio and Schildkraut (1967).
[e] Raji cells were infected with EBV, and the DNA was extracted after 48 hours of infection.
[f] Human lymphocyte line obtained from Dr. W. Henle.
[g] Human lymphocyte lines obtained from the John L. Smith Memorial for Cancer Research, Pfizer Co.

is equivalent to 80 CMV genomes per cell. The counts of cRNA hybridized to 0.1 μg of DNA in 3882 cpm, which is equal to 48 cpm/genome/cell. By this calibration, we should be able to detect as few as two viral genome equivalents per cell.

Typical results of cRNA–DNA hybridization applied to the detection of EBV genomes in human tissue are shown in Table I. P3HR1 cells (virus-producing Burkitt-lymphoma cell line) showed 680 EBV genome equivalents per cell. When the cells were incubated at 32°C for 10 days, the number of genomes increased. A P3HR1 cell line that was no longer positive for EBV capsid antigens by immunofluorescence (IF) test was available, and DNA from these cells was extracted and tested. The number of genome equivalents was drastically reduced to 32 per cell, but virus-specific DNA was still present.

Raji cells, a line of Burkitt lymphoma that does not produce EBV or viral antigens, had been reported to contain EBV DNA (zur Hausen and Schulte-Holthausen, 1970). This observation was confirmed by cRNA–

DNA hybridization, but about ten times more genomes per cell were found. zur Hausen and Schulte-Holthausen (1970) assumed in the calculations from the DNA–DNA hybridization tests that all of the DNA on a filter was hybridized at the saturation level of added [^{3}H]DNA, which probably led to the underestimates.

2. *Messenger RNA–DNA Hybridization*

The RNA–DNA filter hybridization techniques can be used to detect and also to select for virus-specific RNA. Hybridization is carried out with immobilized purified viral DNA on the filter. The amount of viral DNA used is totally dependent on the purpose of the experiment. Reaction conditions, including 50% formamide at low temperature, are more favorable to select for viral mRNA with minimum breakdown of the macromolecules. The radioactive mRNA hybridized to viral DNA on the filter can be eluted by elevation of the formamide concentration in low salt and precipitation with alcohol in the cold. Messenger RNA–DNA hybridization on filters is still the most popular technique to examine the size of viral message in various gradients.

3. *Multisampled DNA Concentration Curves (cRNA–DNA Hybridization on Filters)*

Complementary RNA–DNA hybridization is customarily carried out with a single concentration of the DNA to be analyzed. It is feasible, however, to conduct an analysis in special cases with multiple concentrations of the test-cell DNA. This procedural variation affords some increase in sensitivity when relatively small amounts of hybridizable DNA are present. In the illustration presented, there is approximately a 2- to 3-fold increase in sensitivity. In this example, tissue from a Burkitt's lymphoma without detectable EBV DNA by the conventional cRNA–DNA hybridization was analyzed more closely. Different concentrations of control-cell DNA (HEp 2) and DNA from the lymphoma (FM) were fixed onto nitrocellulose filters and exposed to the same amount of cRNA (Fig. 2). Although the background hybridization increased with higher concentrations of DNA, hybridization for HEp-2 DNA and the degree of hybridization for the lymphoma did not differ significantly with concentrations of up to 200 μg of cellular DNA. The results indicated clearly that there was less than one EBV genome equivalent per cell.

4. *Competition Hybridization*

This is a useful technique to compare quantitatively and qualitatively species of viral RNA as well as to define sequence of gene transcription. There are two methods of approach: (i) simultaneous hybridization competition in which hybridization experiments are performed by incubation of DNA-containing filters with a constant saturation amount of radioactive RNA and increasing amounts of unlabeled competitive RNA; and (ii) sequential competition in which hybridization experiments are carried out sequentially by preincubating the viral DNA filter with increasing amounts of unlabeled competitive RNA for 12 hours and then adding a saturating amount of radioactive RNA. The use of saturating amounts of radioactive indicator RNA in competition experiments is essential. Saturation curves determined with the same batch of DNA filters and radioactive RNA are also essential. Representative examples of the application of hybridization competition can be found in Oxman *et al.* (1971) and Levine *et al.* (1973) in an adenovirus 2-SV40 hybrid system and in Huang *et al.* (1972) in an SV40 system.

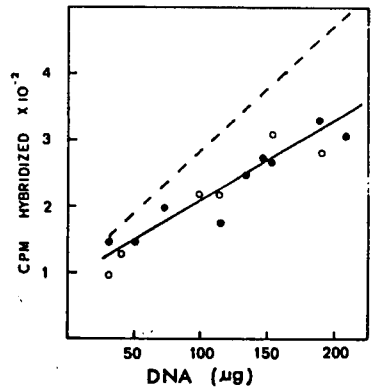

FIG. 2. Comparison of hybridization efficiency between HEp-2 DNA and DNA from the single Burkitt's lymphoma without detectable EBV DNA. Each amount of HEp-2 DNA or the lymphoma (FM 1143) DNA was fixed on a nitrocellulose membrane filter and hybridized with 1.5 × 10^3 cpm of cRNA specific to EBV DNA. (○—○) FM DNA; (●—●) HEp-2 DNA; (——) one genome per cell. (From Nonoyama *et al.*, 1973.)

D. RNA–DNA HYBRIDIZATION IN LIQUID MEDIUM

RNA–DNA hybridization can be performed in liquid without immobilization of DNA on solid support. This technique was introduced by Nygaard and Hale (1964). The handicap implicit in the technique is self-annealing of the DNA in liquid which prevents the hybridization of RNA to the homologous DNA if the ratio of RNA to its homologous DNA sequences is too low. The technique is at its best when separated strands of DNA can be used, for example, in SV40 and adenovirus systems (Sharp *et al.*, 1974; Flint

et al., 1975, 1976a,b). Self-annealing of DNA is obviated, and the RNA–DNA duplexes formed as the result of hybridization can be treated with RNase to remove unhybridized radioactive RNA and precipitated with TCA to quantitate the RNase-resistant counts in the hybrid. Alternatively, hydroxyapatite chromatography can be used to separate the duplexes from single strands of DNA and RNA.

E. COMPLEMENTARY RNA–DNA CYTOHYBRIDIZATION *in Situ*

In the variety of cRNA–DNA hybridization known as cytohybridization *in situ*, hybridization of the cRNA is carried out in fixed cells rather than to extracted cellular DNA (Gall and Pardue, 1971; Jones and Corneo, 1971; Huang *et al.*, 1973; Pagano and Huang, 1974). The great advantage of this technique is its ability to localize virus-specific DNA according to cell type or intracellular location by autoradiography and observation with light microscopy. Essentially similar techniques have been used to localize virus-specific RNA with radiolabeled DNA probes. Potentially it should be possible to extend these methods by the use of high resolution techniques that have been perfected for direct autoradiographic approaches.

General Description of Method. Cells in suspension, exfoliated cells, or tissue sections containing virus-specific DNA sequences are fixed on a slide r cover slip and exposed under controlled conditions to alkali or heat so that the cellular DNA is partially denatured *in situ*. The crucial aspect of this step is avoidance of excessive damage to the cell structure. It is then possible to use either tritiated or iodinated (Shaw *et al.*, 1975) cRNA hybridization directly to the cell preparation or tissue section; this is done under standard buffer conditions for essentially the same period of time used to conduct hybridization on membrane filters. The steps that follow include repeated rinsing and treatment with RNase to remove the nonspecifically adherent cRNA. Autoradiography follows.

Cytohybridization is not as sensitive as cRNA–DNA hybridization on membrane filters in which the DNA from many cells is pooled for hybridization. For example, with EBV DNA sequences in the nonvirus-producing Raji lymphoblastoid cell line, the 60 genome equivalents per cell that these cells contain is well above the limit of detectability by hybridization on membrane filters, i.e., two genome equivalents per cell. By cytohybridization with the same cRNA probes, however, only about 10–15 grains can be seen after a 4-week exposure time (Pagano and Huang, 1974). The number of grains is slightly higher than the number found in the background. If, however, most of the viral genetic material were confined to a few cells, then such cells would stand out, and the test would become relatively sensitive. It is not quite a quantitative test for determination of the amount

of viral genome present, but it is quantitative in the sense that the percentage of cells bearing homologous DNA can be determined. As indicated before, the test has the special advantage of permitting localization of the viral DNA, both with respect to cell type and subcellular site.

This procedure has been used with considerable success in studies of infection with SV40, EBV, human CMV, and adenoviruses. Recent methods that employ iodinated cRNA (Shaw *et al.*, 1975) and scintillation fluor (Huang *et al.*, 1976b, and described below) greatly reduce the exposure time required for *in situ* cytohybridization. Although with ^{125}I the grain size on the autoradiograms is larger, the results by light microscopy are still acceptable.

This procedure for cytohybridization *in situ* is adapted from that of Gall and Pardue (1971) and Jones (1970) with modification (Huang *et al.*, 1973).

Cells or nuclei are pelleted by centrifugation at 1000 rpm (about 100 *g*) for 10 minutes and resuspended in hypotonic solution (0.1 × SSC or Hanks' solution) for 20 minutes at 37 °C; this step is omitted for examination of cytoplasmic viral DNA or RNA. The pellet is resuspended and pelleted at the same centrifugal force and then fixed with freshly prepared ice-chilled ethanol (3 parts) and acetic acid (1 part) for 15 minutes. The cells and nuclei are spun off and washed once with the same fixative. The cells are gently dispersed in fixative with 0.2 ml of the fixative left after centrifugation at low speed. The cell suspension is then spread onto a clean precooled slide and dried in air or quickly in a flame.

In tissue obtained at autopsy or biopsy, tissue blocks are embedded in Ames O.C.T. compound and sliced into sections 0.6–10 μm in thickness and then applied to the slide and fixed with freshly prepared ice-chilled fixative for 15 minutes. After fixation, the slides are dipped in 90% alcohol and absolute alcohol to remove the residual acetic acid, and then dipped into 0.4% agarose at 60 °C and air dried to form a thin agarose layer on the slide to prevent detachment of cells during alkalization and hybridization.

The denaturation of DNA is carried out by alkalinizing the specimens in 0.07 *N* NaOH for 3 minutes; an alternate method of treatment with 0.2 *N* HCl for 20 minutes has been used by McDougall *et al.* (1972). The slides are then washed extensively with 70% alcohol and absolute alcohol and air dried.

One-tenth of a milliliter of [^{3}H] (or ^{125}I) cRNA (3×10^5 cpm/0.1 ml with 1 mg yeast RNA, 0.1% SDS, and 6 × SSC) is applied to each slide and covered with a cover slip to prevent evaporation. The specific activity of the [^{3}H]RNA is about 1×10^7 cpm/μg and of the [^{125}I]RNA about 4×10^7 cpm/μg (Shaw *et al.*, 1975). The hybridization is carried out in a moist chamber at 66 °C for 20 hours. The slides are rinsed with 2 × SSC four times and treated with 40 μg/ml pancreatic RNase for 30 minutes at 37 °C. After

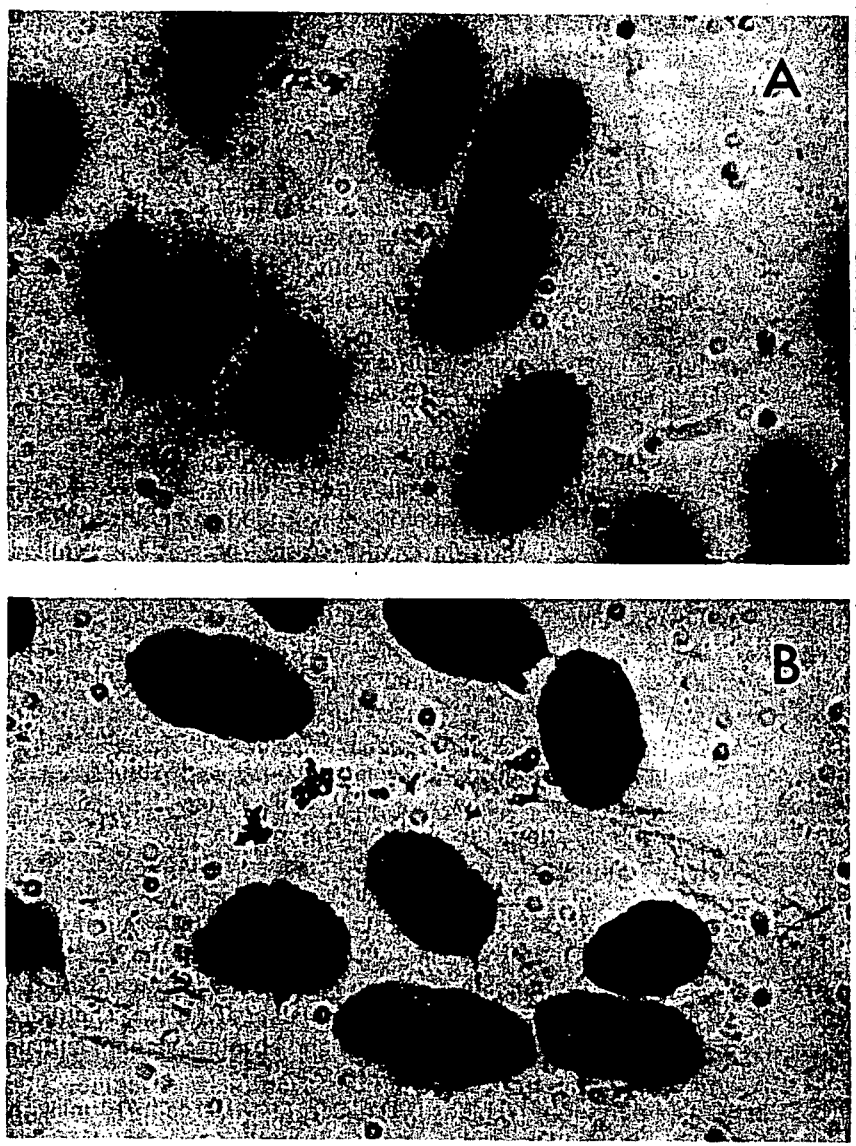

FIG. 3. Cytohybridization *in situ* for the detection of cytomegalovirus DNA: time course of appearance of viral DNA in infected cells. WI-38 cells on cover slips were infected at a high multiplicity (1–2 PFU/cell). The nuclei were released by hypotonic treatment and fixed. The DNA was denatured with 0.07 *N* NaOH for 3 minutes. After washing and fixation cRNA–DNA hybridization was carried out with an input of 5×10^5 cpm CMV cRNA in 0.1 ml per cover slip. After 22 hours of hybridization at 66°C the preparations were rinsed and treated with RNase and then extensively washed with 2 × SSC; autoradiography was carried out with NTB 2 emulsion. (A) Uninfected control cells. (B) Seven hours after infection. (C) Twenty-four hours after infection. (D) Thirty-two hours after infection. (From Pagano and Huang, 1974.)

extensive washing again with 2 × SSC, the slides are sequentially dehydrated with 70%, 95%, and absolute alcohol and dried.

Kodak Nuclear Track NTB 2 is used for autoradiography. The exposure time for tritiated labeled material ranges from 4 weeks to 2 months, but for ^{125}I material the time is overnight to one week; the time depends mainly on the specific radioactivity of viral cRNA and also on the viral DNA content of the cells. Recently we have been able to shorten the exposure time of ^{3}H-labeled material from 4 weeks to 1 or 2 days by dipping the emulsion-covered slide (after complete drying) in liquid scintillation fluid—dioxane and PPO (1% w/v) and POPOP (0.02%). The slides are developed in Kodak D-19 developer for 3 minutes, gently rinsed with water for 30 seconds, and then fixed in Kodak rapid fixer for 2–3 minutes. After rinsing in water and drying in air, the slides are stained with Giemsa for 30 minutes.

Figure 3 shows an example of *in situ* RNA–DNA cytohybridization used to observe the time course of viral DNA synthesis in human CMV-infected cells. Under the conditions of the experiment, viral DNA synthesis commenced in these cells 20–24 hours after infection and reached its peak 70 hours after infection (Huang *et al.*, 1973). The first indication of hybridization, found 7 hours after infection, is almost certainly the input virus. However, within 24 hours after infection, replicated CMV DNA is found in two acrocentric areas rather than diffusely scattered through the nuclei. Only nuclei are shown since hypotonic treatment was used, but if preparations of whole cells were used, then it would be possible to show the transition of viral genetic material from intranuclear to cytoplasmic sites.

Figure 4 shows an example of cRNA–DNA cytohybridization *in situ* with CMV[^{3}H]cRNA applied to kidney tissue (in collaboration with Dr. G. Nankervis). The tissue was from autopsied kidney of a congenitally infected infant with CMV infection. This photograph does not represent the incorporation of [^{3}H]thymidine in the DNA, but rather the hybridization of CMV[^{3}H]cRNA to the viral DNA in the cells. The grains which deposit heavily in cuboidal epithelial cells in the collection tubules represent the localization of viral DNA. As shown before (Huang *et al.*, 1976b), CMV viral structural antigen was also detected in these cells by the ACIF test.

The application of cytohybridization techniques to EBV and SV40 systems is shown in Figs. 5 and 6.

Cytohybridization is also potentially useful for the detection and localization of virus-specific mRNA and has been used for this purpose by McDougall *et al.* (1972) and Dunn *et al.* (1973). For the detection of virus-specific mRNA, it would be necessary to use radioactively labeled pure viral DNA. There are two problems: (1) Viral DNA of high specific activity is required; viral DNA of sufficiently high specific radioactivity is now available from *in vitro* labeling techniques (either tritiated or [^{125}I]DNA). (2) An enzymic

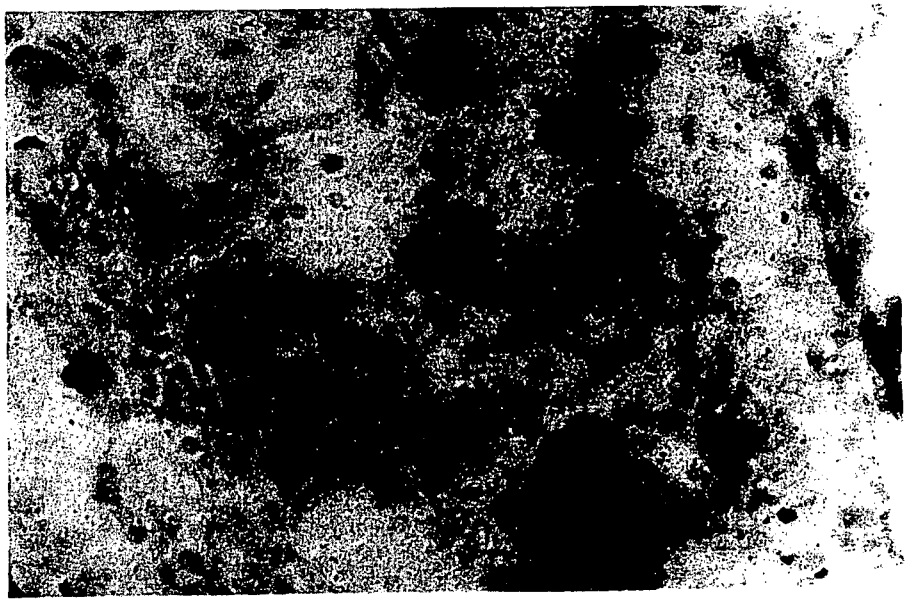

FIG. 4. Complementary RNA–DNA cytohybridization *in situ* with CMV[^{3}H]cRNA applied to kidney. A tissue block from the kidney of a congenitally CMV-infected infant was embedded in Ames O.C.T. compound. The tissue was sliced into sections 6 μm thick, transferred to slides, and fixed with freshly prepared fixative. After dehydration the section was exposed to 0.07 *N* NaOH for 3 minutes and dehydrated with 70% and 95% ethyl alcohol. The cytohybridization was carried out as described in the text. (From Huang *et al.*, 1976b.)

means of hydrolyzing residual labeled DNA, equivalent to RNase treatment, without destruction of duplex molecules is necessary. For this purpose single-strand-specific nuclease, such as S1 enzyme from *Aspergillus oryzae*, or the nuclease with similar properties from *Neurospora crassa* have proved to be suitable.

F. DNA–DNA RENATURATION KINETICS ANALYSIS*

1. *Practical Theory*

In aqueous solution single-stranded complementary segments of DNA will reassociate and form double-stranded hydrogen-bonded structures (Britten *et al.*, 1974; Britten and Kohne, 1968). Reassociation is generally

*Modified from Shaw and Pagano (1976).

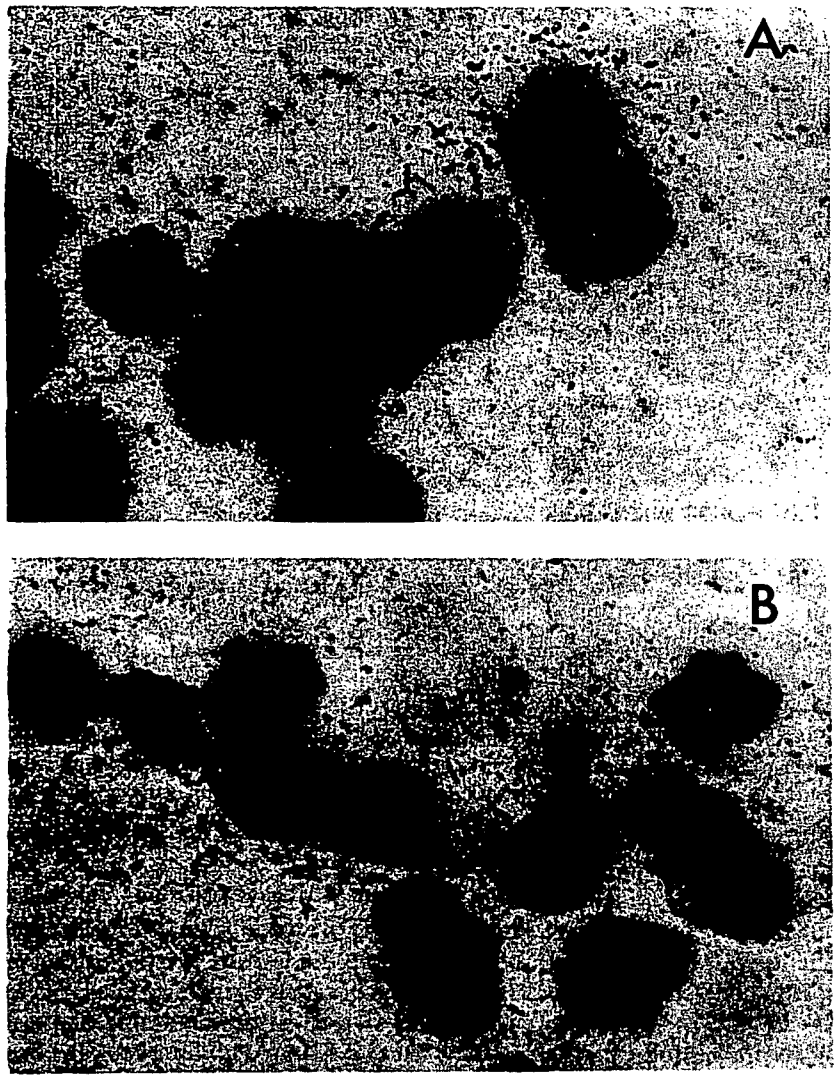

FIG. 5. Cytohybridization with EBV cRNA to the virus-producing (HR-1) line and a non-us-producing line (Raji) or Burkitt's lymphoma cells. The HR-1 or Raji cells were washed d treated with 0.1 × SSC for 20 minutes at 37°C. The cells were then fixed and applied to the de for cytohybridization. The amount of EBV [^{3}H] cRNA applied to each slide was 5 × 10^5 m in 0.1 ml 6 × SSC with 1 mg of yeast RNA and 0.1% SDS. (A) The virus-producing cell e HR-1 (exposure time 2 weeks). A heavy deposition of grains was concentrated in the us-producing cells. This indicates the heterogeneity of HR-1 cells. (B) The nonvirus-oducing cell line Raji (exposure time 4 weeks). A scattering of grains higher than background is found in the nuclei of Raji cells; Raji cells contain 60 EBV genomes per cell, located in the romosomes, and distributed uniformly among the cells. (From Pagano and Huang, 1974.)

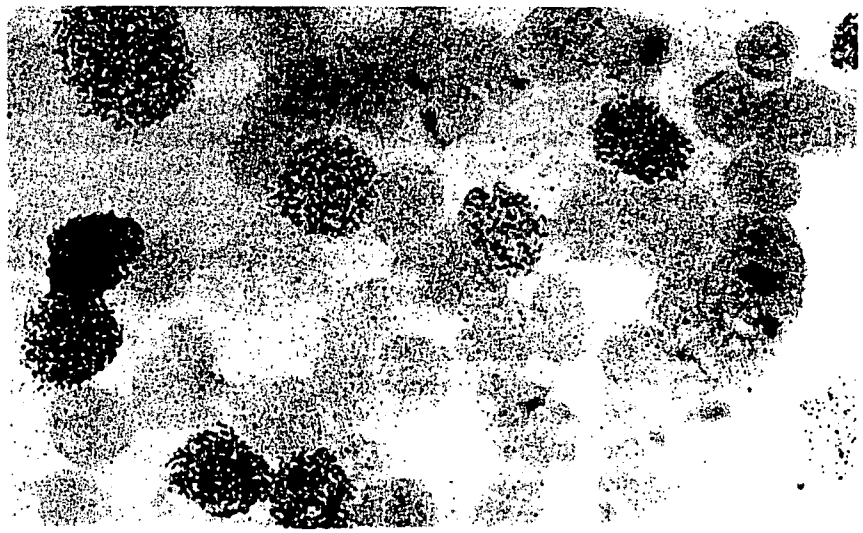

FIG. 6. SV40-transformed WI-38 human fibroblasts—WI-38Va13. The cells grown on a cover slip were hypotonically treated and fixed with fresh fixative as described in the text. SV40 cRNA of specific activity 1 × 10^7 cpm/μg was used for cytohybridization, and the exposure time was 3 weeks. There is a striking difference in the distribution of the hybridizable SV40 DNA in the nuclei. WI-38 cells are partially permissive for the replication of SV40. The phenomenon of active SV40 DNA replication in these transformed cells may be similar to that for EBV in the HR-1 cell system (×1000). (From Pagano and Huang, 1974.)

carried out in dilute solution so that it is possible to follow the kinetics which are typically those of a second-order rate reaction. The kinetics are significantly influenced by temperature and ionic milieu. The rate-limiting step is an in-register collision at one or more sites along pairs of single-stranded complementary segments of DNA (Wetmur and Davidson, 1968). The equation for an ideal second-order reaction is $C/Co = 1/(1 + KCot)$, where C is the concentration of a single-stranded DNA, Co is the total DNA concentration, K is the reassociation rate constant, and t is time (Britten *et al.*, 1974). The units for C and Co are moles of nucleotides per liter; t is in seconds. The reassociation rate constant K depends on the incubation conditions and complexity of the DNA, and it is the reciprocal of the $Cot_{1/2}$ value, defined later. The rate of reassociation depends on DNA complexity and concentration, length of the single-stranded segments, viscosity, temperature, and salt concentration. When these variables are standardized, the reaction rate becomes directly dependent on DNA concentration.

Since all of the DNA in the reaction mixture is initially denatured and, therefore, present as single strands at the beginning of the reaction, the extent of reassociation is determined at any time during incubation by measurement of the amount of double-stranded DNA formed. This determination requires discrimination of single- and double-stranded DNA. Hydroxyapatite is used extensively for this purpose because of its capacity to retain double-stranded DNA relatively exclusively at low (0.14 *M* potassium phosphate), but not at high (0.4 *M*) salt concentrations (Kohne and Britten, 1971). Another method for the discrimination of double-and single-stranded DNA is by digestion of single-stranded DNA remaining in the reaction mixture by a single-strand specific nuclease, S1 enzyme (Ando, 1966), and nuclease from *Neurospora crassa*. Ideally, digestion with such nucleases leaves only double-stranded DNA precipitable from the reaction mixture. If the DNA has been labeled with a radioactive precursor, then the amount of duplex DNA formed can be inferred by scintillation spectrometry.

The results of a reassociation experiment may be expressed in terms of *Co/C* versus time if a linear representation of the data is desirable, or as the percentage of double-stranded DNA formed versus time of incubation. In the latter case, it is convenient to plot the time scale as the product (*Cot*) of total nucleic acid concentration, *Co* (moles of nucleotides per liter), and time *t* (seconds). Plots of data in terms of *Cot* are useful for estimating the completion of the reaction and for comparing DNAs from various sources. When different DNAs are compared, it is usually at their $Cot_{1/2}$ value, the value obtained when 50% of the single-stranded radioactive probe DNA has reassociated.

2. *Factors Influencing the Rate of Reassociation*

Several such factors have been described in detail by Wetmur and Davidson (1968). The main points can be summarized as follows:

(a) Effect of temperature on the rate of reassociation. The rate of reassociation has a bell-shaped dependence on temperature with a region of maximum reassociation around $T_m - 25$°C. The reassociation rate reaches a broad plateau from 15°C to 30°C below T_m; it drops significantly when the reaction temperature is lower than 30°C below T_m or higher than 15°C below T_m.

(b) Effect of DNA complexity on the rate of reassociation. The rate of reassociation is inversely proportional to the complexity or number of base pairs (or molecular weight) in the nonrepeating DNA complement of the viral or cellular genome. The DNA should be sheared to a homogeneous size, 300–400 nucleotide pairs.

(c) Effect of pH. Within the pH range of 5–9, the rate of reassociation in 0.4 *M* sodium ion is essentially independent of pH. There is a remarkable decrease in the rate of reassociation when the pH is above this range.

(d) Effect of ionic strength and viscosity. The rate of reassociation is dependent on the ionic strength below 0.4 *M* Na^+; there is almost a 7-fold increase in the rate of reassociation when 0.4 *M* Na^+ is used as compared with 0.15 *M*. The rate is almost independent of salt concentration when ionic strength is above 0.4 *M*. The differences in the rate of reassociation at 0.4 *M* and 1.0 *M* is 2-fold.

Since the rate of reassociation is proportional to the frequency of mechanical collision of the two complementary DNA segments, the rate of reassociation decreases with increasing solvent viscosity. Because of this, the viscosity should be properly controlled.

3. *Uses and Procedure*

Reassociation curves can establish whether DNA contains repeated sequences (a rapidly reassociating fraction), as is characteristic of eukaryotic DNA, or whether it is unique DNA (DNA for which repeated sequences are not recognized). One of the most useful applications of reassociation experiments has been in the area of viral oncology where it has been possible to detect small numbers of copies of viral genomes in the DNA isolated from cells suspected of carrying viral information, but in which biologically active virus is not demonstrable. It is possible to detect as little as 0.1–0.2 viral genome per cell in some instances. The sensitivity of the assay depends on the use of viral probes which have been labeled to high specific radioactivity.

In a typical reassociation experiment, purified viral DNA, labeled during virus replication or by *in vitro* techniques, is mixed with a large excess of unlabeled test (cellular) DNA; DNA with no detectable sequence homology to the labeled probe would be selected as a substitute for the test DNA in a control reaction mixture. The DNAs are sheared to uniform size, denatured, and then the mixtures are adjusted to the proper incubation conditions. At various times during incubation aliquots are removed from the mixtures to determine the amount of double-stranded DNA formed. At the end of the incubation period, the reassociation rates are compared. If the test DNA lacks viral DNA sequences, then the reassociation rates of the test and control reactions would be the same. The rates would be the same because the concentration of viral DNA in each mixture is identical. If unlabeled viral DNA sequences are present in the test reaction, then the total viral DNA concentration would be greater than that of the control reaction. This would increase the rate of reassociation of the labeled DNA in the test reaction over the labeled DNA in the control reaction and would indicate that viral DNA sequences are present in the test DNA.

Outlined below is a technique which utilizes DNA–DNA reassociation kinetics to detect the presence of EBV DNA in the DNA of two B-lymphoblastoid cell lines; Raji, a nonvirus-producing cell line containing 50–60

copies of viral DNA per cell, and P3HR-1, an EBV-productive cell line harboring 800–1000 copies of viral DNA per cell.

Stock solutions of Raji, P3HRl, calf thymus, and ^{3}H-labeled EBV DNA free of protein and RNA are sonicated. The DNA is concentrated to 10 mg/ml (Raji, P3HRl, and calf thymus DNA) or 2×10^{-2} μg/50 μl (EBV DNA) in neutralized 2.5 m*M* EDTA, and dialyzed extensively against the same solution. The solutions are stored at −20°C until used. To demonstrate the effect of DNA concentration on the rate of reassociation, four reassociation mixtures are assembled at room temperature, as shown in Table II: a control containing calf thymus DNA, two concentrations of Raji-cell DNA, and one concentration of P3HR1 DNA. Because viscosity affects the rate of reassociation, all mixtures are adjusted to the same final DNA concentration by adding sonicated calf thymus DNA.

All reaction components are added except NaCl. The mixtures are heated for 10 minutes in a boiling water bath and quick-chilled in ice water. NaCl is then added, and each mixture is divided into 9 or 10 fractions by withdrawal of 0.1 ml of the mixture into the center of a 100 μl pipette. The pipettes are sealed by flame and are placed in a water-filled tube which is immersed in a 67°–70°C water bath. At various times during incubation, fractions are removed and frozen at −20°C.

At the end of the incubation period, each sample is added to 1.9 ml of S1 digestion buffer; then each mixture is split into two 1-ml fractions, one to be digested with S1-nuclease, the other to serve as a control. Five to ten microliters of S1-nuclease are added to one tube of each set. After 2–3 hours at 40°C, each sample is chilled to 0°C and mixed with 0.2 ml of ice cold 100% TCA to precipitate undigested DNA. The precipitates are collected on Millipore filters by suction and washed twice with cold 5% TCA.

TABLE II
REASSOCIATION KINETICS ANALYSIS OF EBV DNA[a,b]

	[^{3}H]EBV DNA	Calf thymus DNA	Raji DNA	P3HRl DNA	Reassociation buffer	H_2O	6 *M* NaCl[c]
Control	0.05	0.20	—	—	0.1	0.40	0.25
Raji	0.05	0.16	0.04	—	0.1	0.40	0.25
Raji	0.05	0.12	0.08	—	0.1	0.40	0.25
P3HRl	0.05	—	—	0.20	0.1	0.40	0.25

[a] From Shaw and Pagano (1976).
[b] Figures in milliliters.
[c] Added after heat denaturation.

The filters are dried at room temperature and counted in a toluene-based scintillation fluid.

To determine the fraction of double-stranded DNA present in each 100 μl sample, the ratio of cpm of the digested and undigested portions is determined. The value is expressed as a percentage. The results of reassociation of ^{3}H-labeled EBV DNA are shown in Fig. 7 and are plotted as the percentage of renatured DNA versus *Cot*. The *Cot* values are in terms of [^{3}H]EBV DNA. The time of incubation is also shown. It is evident from the $Cot_{1/2}$ value of each curve that the reassociation rate increases as the concentration of lymphocyte DNA increases. The increase in reassociation rate is due to the presence of viral DNA sequences in the lymphocyte DNA preparations.

4. *Problems*

To avoid some of the pitfalls which accompany reassociation experiments, several precautions can be taken (see Britten *et al.*, 1974). If it is

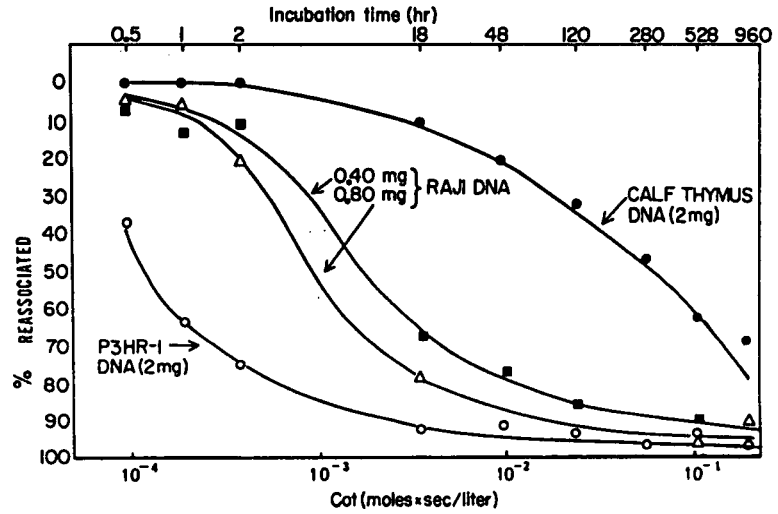

FIG. 7. Reassociation of ^{3}H-labeled EBV DNA. Each reaction contains per milliliter: 0.02 μg [^{3}H]EBV DNA, 2 mg calf thymus DNA (●—●); 0.02 μg [^{3}H]EBV DNA, 0.8 mg Raji DNA, 1.6 mg calf thymus DNA (■—■); 0.02 μg [^{3}H]EBV DNA, 0.8 mg Raji DNA, 1.2 mg calf thymus DNA (△—△); 0.02 μg[^{3}H]EBV DNA, 2 mg P3HR1 DNA (○—○). ^{3}H-labeled EBV DNA is 2×10^6 cpm/μg. The DNA in each reaction was denatured and incubated at 70°C in buffered 1.5 *M* NaCl. At the time indicated 0.1 ml fractions were removed and the amount of double-stranded DNA in each was determined. The percentage of single-stranded DNA which reassociated is plotted versus *Cot* and incubation time. (From Shaw and Pagano, 1976.)

possible to prepare labeled DNA *in vivo* with a high specific activity, then many of the problems associated with *in vitro* labeling of DNA can be avoided. Probes prepared *in vitro* should be characterized before use by hydroxyapatite chromatography and by a single-strand-specific nuclease. Each new batch of hydroxyapatite and each new preparation of single-strand-specific nuclease should be tested with a DNA whose structure has been well established. DNA fragments of different lengths reassociate at different rates. Therefore, in order to obtain controlled rates of reassociation, it is important to control the shearing procedure. This can be accomplished by routine sizing of the sheared DNA fragments. Sizing is also recommended if overdigestion with DNase is suspected.

Although the chelating agents help to protect DNA during reassociation, their presence can interfere with the separation of single- and double-stranded DNA on hydroxyapatite. If S1-nuclease is used instead of hydroxyapatite, the concentration of $ZnCl_2$ should be in excess of the chelator, since Zn^{2+} is necessary for S1 activity. The presence of sodium phosphate in concentrations as low as 10 m*M* can inhibit S1 activity.

Because of the possibility of concentrating salts with DNA during ethanol precipitation, stock solutions of DNA should be dialyzed against buffer before heat denaturation. This ensures that the temperature used for denaturation is well above the melting point of the DNA.

G. Partial Homology: Detection by DNA–DNA Renaturation Kinetics

In addition to its utility as a sensitive and quantitative assay for amounts of homologous DNA, renaturation kinetics analyses are also of value for the determination of degree of homology between related but not identical genomes. With present technology, this technique is applicable if there is at least 10% homology between the genomes being compared. For lesser degrees of homology, heteroduplex formation provides a more sensitive method; this technique can be used to detect as little as 5% homology.

Examples of reciprocal analyses of radiolabeled EBV and human CMV genomes are shown in Figs. 8 and 9. These results indicate a lack of detectable homology between the AD169 strain of HCMV and the P3HR1 strain of EBV. Had there been some degree of homology between the unlabeled DNA under test and the labeled probe DNA, an initial acceleration of renaturation of the index DNA would have been produced. Since the variation around the control points is not large in these tests, it is clear that in the neighborhood of 5–10% homology in the added unlabeled test DNA would produce a perceptible acceleration of renaturation of the labeled DNA.

Epstein–Barr virus DNA sequences are found in cell lines derived from

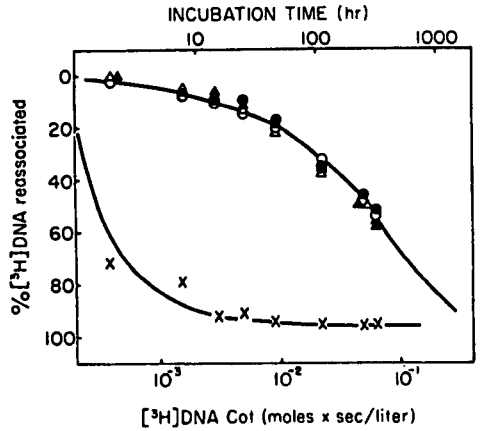

FIG. 8. Reassociation kinetics analysis of tritiated human cytomegalovirus DNA with the DNA of HSV 1, HSV 2, and EBV. Sonically disrupted ³H-labeled CMV DNA (strain AD-169), 0.02 μg (3.4 × 10⁴ cpm), 20 μg of calf thymus DNA, and 4 μg of unlabeled viral DNA were mixed and denatured in the presence of 0.01 *M* Tris-HCl (pH 7.4) and 0.0025 *M* EDTA. The salt concentration was then adjusted to 1.2 *N* NaCl. The hybridization was carried out at 66°C, and the fraction of reassociated [³H]DNA was analyzed by S1 enzyme differential digestion as described above. Calf thymus control DNA, 4 μg (○); HSV 1 DNA (△); HSV 2 DNA (▲); EBV DNA (●); and AD-169 DNA (×). (From Huang and Pagano, 1974.)

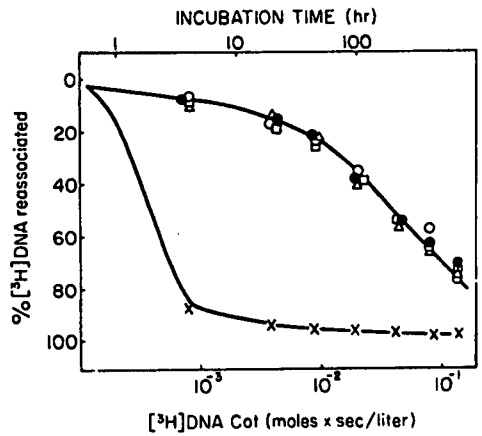

FIG. 9. Reassociation kinetics and analysis of tritiated EBV DNA with the DNA of HSV 1, HSV 2 and CMV DNA. Sonically disrupted ³H-labeled EBV DNA, 0.02 μg (5.4 × 10⁴ cpm), 20 μg of calf thymus DNA and 3 μg of unlabeled viral DNA were mixed. The experiment was carried out as described in Fig. 8. Calf thymus DNA (○); HSV 1 DNA (△); HSV 2 DNA (●); AD-169 DNA (□); and EBV DNA (×). (From Huang and Pagano, 1974.)

patients with infectious mononucleosis (IM). The question of whether the EBV genomic material found in Burkitt's lymphoma and in infectious mononucleosis are in fact identical has been a persistent one. Results shown in Fig. 10 indicate clearly that 25–35% of the DNA sequences found in the P3HRI-derived EBV cannot be detected in the four cell lines derived from two patients with infectious mononucleosis. These results do not distinguish between the presence of an extensively deleted EBV genome in the IM cell line as against an "IM strain" of EBV with up to 35% heterologous DNA sequences. In order to discriminate between these possibilities, it would be necessary to prepare radiolabeled IM EBV DNA from purified IM virus and to conduct a reciprocal type of DNA–DNA renaturation kinetics analysis with cold P3HRI EBV DNA. At present sufficient quantities of IM EBV are not available. Another approach would be to determine the molecular weight of the IM virus DNA by sedimentation analysis or contour length measurements by electron microscopy. Ultimately it will be possible to explore homology between the genomes derived from these two sources by heteroduplex formation (see Section IV,B). A similar lack of homology ranging up to 40% has been found in analysis of homologous DNA sequences contained in nasopharyngeal carcinomas from Tunisia in comparison with the HRI EBV genome (Pagano *et al.*, 1975; J. S. Pagano and J.-L. Li, unpublished data).

IV. Special Methods

A. Specific DNA Fragments and Blot-Transfer Hybridization

Restriction endonucleases have become powerful tools for analysis not only of smaller viral genomes but also of genomes of increasing complexity and molecular size. Cleavage of DNA into specific terminal fragments and construction of a DNA fragment map provide elements needed for detailed characterization of the viral genome, for the determination of the direction of DNA replication, and for the regulation of gene transcription. The sensitivity of detection of viral genetic material in various tumor and virus-transformed cells by DNA–DNA hybridization can be enhanced by the use of specific DNA fragments as probes. Location of viral mRNA on a genetic map can also be approached by hybridization of the mRNA, isolated from lytically infected or transformed cells, to restriction endonuclease-generated fragments.

There are several ways of recovering the specific viral DNA fragments. The DNA is first subjected to restriction enzyme digestion, and the DNA

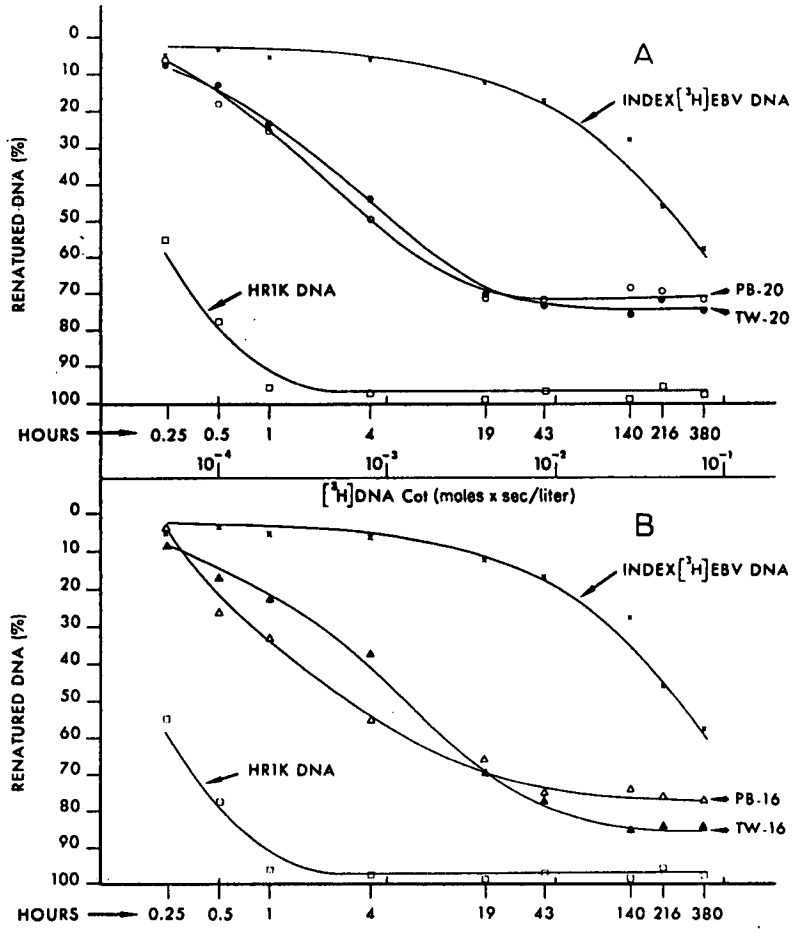

FIG. 10. Incomplete homology of the EBV genome of the viral DNA sequences in lymphocyte lines from infectious mononucleosis. The determinations were by DNA–DNA renaturation kinetics analyses. (A) [³H]EBV DNA (0.02 μg) + calf thymus DNA (2 mg); PB20 DNA (1.1 mg) is from a cell line derived from peripheral blood; TW20 DNA (1.8 mg) is a cord-lymphocyte line transformed by throat washings from the same patient; HRIK DNA (2 mg) is homologous DNA from the EBV-producing cell line that was the source of the index EBV DNA. (B) PB16 DNA (1.5 mg) is from a cell line established from the peripheral blood of another patient with infectious mononucleosis; TW16 DNA (1.5 mg) was from a cell line produced by exposure to throat washings from the same patient.

Radiolabeled EBV DNA (0.02 μg, specific activity of 1.8×10^6 cpm/μg) is present in all renaturation mixtures. The total concentration of DNA in all mixtures was brought to 2 mg by the addition of calf thymus DNA. (From Pagano *et al.*, 1976.)

fragments are then electrophoresed to separate them according to size. The DNA fragments can be located in agarose gels by staining with 0.5 μg/ml of ethidium bromide and observation under UV light or by ^{32}P autoradiography (Fig. 11). The DNA fragments can be recovered from the gel either by electrophoretic elution or by potassium iodide solubilization. The solubilization method of Hayward *et al.* (1975) is briefly described as follows. The agarose-gel blocks are dissolved by shaking 5 volumes of saturated potassium iodide solution in TE buffer (0.01 *M* Tris-HCl, pH 8.5; 0.001 *M* EDTA) at 37°C for 20 minutes. DNA-grade hydroxyapatite is added with shaking to the mixture to absorb the DNA. The hydroxyapatite granules which absorb the DNA fragments are then collected by centrifugation. The pelleted granules are washed once with 0.1 *M* potassium phosphate buffer, pH 6.8. Double-stranded DNA is then eluted by 0.4 *M* potassium phosphate buffer, pH 6.8. The eluant containing the DNA fragments is then phenol-extracted once. The EtBr which is intercalated in the double-stranded DNA is removed by hydrogen Form Dowex 50 (Ag 50W-X8, 200–400 mesh, Bio-Rad) in high salt buffer (1 *M* NaCl, 0.1 *M* Tris-HCl, pH 8, and 0.01 *M* EDTA). The DNA is then dialyzed against H_2O and precipitated with alcohol.

Hybridization of viral mRNA to viral DNA fragments has been successfully applied to adenovirus and herpesvirus systems (for reference, see Flint *et al.*, 1976a,b). Assignment of viral mRNA, purified from transformed cells or from different stages of infected cells, to viral DNA fragments, and assignment of isolated specific RNA, e.g., tRNA or 5 S RNA, to a set of restriction endonuclease-generated fragments can be approached by blot transfer and hybridization. This transfer technique is also called Southern's technique (1974). The DNA fragments are denatured *in situ* in the agarose-slab gel by immersing the gel in 0.5 *N* NaOH for 20 minutes. After denaturation, the solution is neutralized with 1.1 *N* HCl in 0.2 *M* Tris. The gel is then immediately transferred onto 4–5 layers of Whatman #1 filter papers immersed in or in contact with 6 × SSC. A sheet of nitrocellulose membrane paper (Millipore) is laid on the gel. Paper towels or Whatman filter papers are put on top of the nitrocellulose filter sheet to absorb the liquid from the gel and the 6 × SSC in which the filter papers under the gel are immersed. The DNA fragments are then transferred to and immobilized on the nitrocellulose by blotting and by capillary diffusion of 6 × SSC. The membrane filter with the set of restriction endonuclease-generated fragments is then dried at room temperature for 3 hours and baked at 80°C in a vacuum oven for 3 hours. The membrane filter is then cut into long strips for hybridization against radioactive mRNA or cRNA; each strip should contain a whole set of DNA fragments. The hybridization is carried out as with RNA–DNA hybridization on membrane filters (Section III,C). The DNA fragments from which mRNA is derived can be detected by autoradiography.

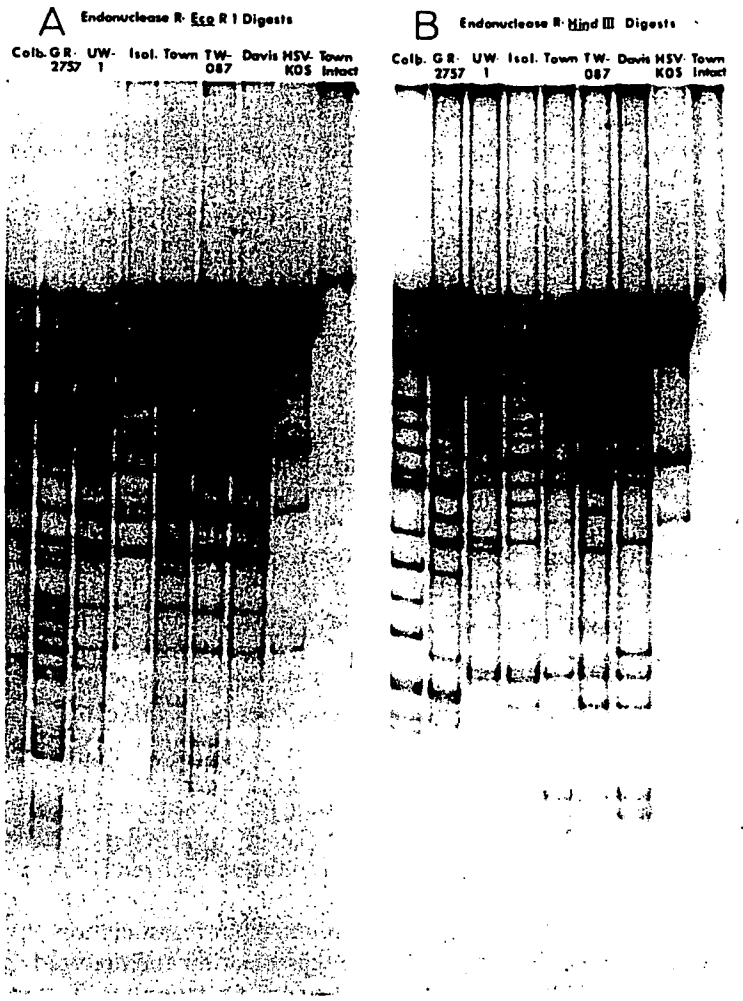

FIG. 11. Comparison and analysis of various CMV strains by cleavage of their DNA with restriction endonucleases EcoR1 and Hind. III. CMV DNA labeled with ^{32}P and purified as described in the text was dissolved in TBS (0.05 *M* Tris-HCl, pH 7.4, and 0.15 *M* NaCl) and digested with either enzyme in the presence of 10 m*M* $MgCl_2$ and 5 m*M* β-mercaptoethanol for 24 hours at 37°C. All samples were electrophoresed on a 1% agarose-slab gel in 1 E buffer until the tracking dye (bromphenol blue) migrated to the bottom of the gel. The gels were dried and exposed to x-ray film as described (Huang *et al.*, 1976b). KOS, a strain of herpes simplex virus, was used as a marker and a control for digestion conditions. (A) EcoR1 digests. (B) Hind III digests. KOS in both panels was digested with EcoR1 enzyme. (From Huang *et al.*, 1976b.)

B. ELECTRON MICROSCOPY: HETERODUPLEX FORMATION AND R-LOOP FORMATION

Heteroduplex formation provides a powerful direct method to localize specific gene sequences in genomes of high complexity and to compare the degree of homogeneity between two gene sets. The general principle, considered in detail by Davis and Davidson (1968) and Davis *et al.* (1971), is that after denaturation two partially complementary or partially noncomplementary strands are allowed to renature under optimal conditions, and the resulting products are observed by electron microscopy for sequence homology. By differentiating single- and double-stranded DNA with electron microscopy in suitable preparations, the regions of homology and nonhomology can be located and mapped. The technique has been widely used in gene localization, for example, mapping the position and size of bacteriophage deletion and insertion mutations (Westmoreland *et al.*, 1969) and localizing SV40 integration sites in adenovirus 7–SV40 hybrid DNA molecules.

The procedures and methods for heteroduplex formation and mounting DNA for electron microscopy have been described in publications of Davis *et al.* (1971). In this technique, summarized from Davis *et al.* (1971), the DNA can be either purified or directly prepared from virus or phage particles by heat or chelation shocking of the phage, or by treatment with urea or $NaClO_4$. The phage protein present does not significantly interfere in the reaction and the electron microscopy (Davis *et al.*, 1971). DNA from two different phages or viruses are denatured by alkali (in 0.5 ml of 0.1 *M* NaOH, 0.02 *M* EDTA) for 10 minutes at room temperature and then neutralized by adding one-tenth volume of 1.8 *M* Tris-HCl, 0.2 *M* Tris-OH. Formamide (99%) in a volume of 0.5 ml is added to the neutralized DNA solution. The final pH of the solutions should be around 7.5–8.5. Under these conditions, 50% of the DNA renatures in 1–2 hours. After reassociation, the reaction mixture is cooled to 0°C to stop the reassociation and dialyzed against 0.01 *M* Tris-HCl, pH 8.5, and 1 m*M* EDTA at 4°C for mounting. Two mounting techniques—the aqueous technique with ammonium acetate and the formamide technique—were described in the original work of Davis *et al.* (1971).

R-loop formation is a technique that makes use of the principle of heteroduplex formation, but with RNA instead of with DNA as in D-loop. RNA is hybridized to double-stranded DNA in the presence of formamide to displace the segment of identical DNA sequence in a genome of high complexity. The resulting triplex structure of an RNA–DNA duplex with single-stranded DNA is called the R-loop; the techniques have been described in detail recently by Thomas *et al.* (1976) and White and Hogness (1977) in a *Drosophila* rRNA (rDNA) system.

R-loop technology can be used to localize genes for early functions in the intact viral genome by hybridizing early mRNA (for example, SV40 early mRNA) to the linear viral genome (in this case EcoRI-digested SV40 DNA). Integrated viral gene sequences can be localized by hybridizing the virus-specific mRNA isolated from virus-transformed cells to the intact linear viral DNA molecule. DNA fragments generated by restriction enzymes also can be assigned locations on the intact genome with the use of cRNA synthesized from fragment templates and R-loop mapping techniques.

The method and buffer used for R-loop formation by Thomas *et al.* (1976) are as follows: 10 μl of a solution containing approximately 0.3 μg each of RNA and DNA, 0.1 *M* NaCl, and 0.05 Tris-HCl, pH 7.5, were added to 50 μl of buffered formamide solution. The buffered formamide was prepared by mixing 0.42 ml of formamide, 50 μl of 1 *M* pipes [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) $N_{1,4}$], pH 7.8, 12 μl of 0.5 *M* Na_3 EDTA, and 18 μl of H_2O. The final formamide and cation concentration were 70% and 0.17 *M*, respectively. The mixture was covered with paraffin oil, sealed in a siliconized glass tube, and heated at 47°C for 20 hours. The sample was then mounted for electron microscopy by the formamide technique of Davis *et al.* (1971).

The rate of R-loop formation, as reported by Thomas *et al.* (1976), reaches its maximum at the temperature at which half of the duplex DNA is irreversibly converted to single-stranded DNA and falls precipitously a few degrees above or below that temperature. Once the R-loop forms, it displays considerable stability; the formamide can be removed, and the DNA can be cleaved with restriction enzyme without loss of the structure. The R-loop is quite sensitive to RNase; incubation in 10 μg/ml of RNase (in 0.1 *M* NaCl, 0.5 *M* Tris-HCl, pH 7.5, 0.1 *M* EDTA) for 5 minutes at 37°C leads to complete loss of R-loops. The loop is also sensitive to alkaline hydrolysis in 0.2 *N* NaOH at 37°C for 10 minutes.

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